Detection of Tumor Cells in the Peripheral Blood of Nonleukemic Patients With B-Cell Lymphoma: Analysis of “Clonal Excess”

By Frances S. Ligler, R. Graham Smith, John R. Kettman, José A. Hernandez, Joyce B. Himes, Ellen S. Vitetta, Jonathan W. Uhr, and Eugene P. Frenkel

Patients with malignant lymphocytic lymphoma often respond to therapy with the disappearance of mass disease and achievement of complete clinical remission. Since the disease usually reappears and eventually causes death, it appears that disseminated disease continues to exist undetected by current methods of clinical analysis. Samples of peripheral blood from nonleukemic patients with malignant lymphocytic lymphoma were examined for an excess of cells bearing the same immunoglobulin light-chain type as tumor cells from tissues involved with the lymphoma. Cells from tumor-bearing tissue and from peripheral blood (which appeared normal by the usual laboratory criteria) were stained with the anti-light-chain reagents, and the fluorescence intensity of individual cells was quantified using the fluorescence-activated cell sorter. Comparison of the number of $\kappa^+$ and $\lambda^+$ cells at each level of fluorescence intensity detected monoclonal populations representing as little as 0.1% of the lymphocytes. Though increases in the proportion of monocytes or null cells to lymphocytes prevented complete analysis of B-cell staining in some cases, all peripheral blood samples from patients with lymphoma that could be analyzed (11/20) exhibited evidence of “clonal excess.” Thus, the recognition of an excess of cells bearing the same light-chain type in both blood and tumor tissue provides strong evidence that the circulating monoclonal lymphocytes were tumor cells.

The recognition of small numbers of tumor cells admixed with normal cells is difficult if the tumor cells have no distinguishing morphological features. We have exploited the monoclonality of B-cell tumors to detect tumor cells in the peripheral blood of nonleukemic patients with malignant lymphoma of the B-cell type. The rationale of these studies is based on the expression of surface immunoglobulins containing only one light-chain type, either kappa ($\kappa$) or lambda ($\lambda$), on monoclonal populations of B cells. In patients with malignant lymphoma and a “spill over” type of leukemia, cells from the solid tumor and blood bear both the same classes of heavy chain and the same light-chain type, providing evidence that circulating and noncirculating tumor cells are derived from the same clone. Teodorescu and Mayer studied patients with chronic lymphocytic leukemia (CLL) and suggested that an abnormal ratio of kappa-bearing ($\kappa^+$) to lambda-bearing ($\lambda^+$) cells in the peripheral blood as assessed by conventional fluorescence microscopy might be used to detect tumor cells in the peripheral blood.

The present study focused on patients with malignant lymphoma whose blood was assessed as normal by the classical techniques. Presumably, if tumor cells were present in the blood, they would be few in number. Therefore, a technique of considerably greater sensitivity than ordinary fluorescence microscopy was required for the detection of a small abnormal population of cells bearing one light-chain type. The fluorescence activated cell sorter (FACS), which quantifies the fluorescence intensity of large numbers of specifically stained cells, provides such a technique. Since cells from most lymphomas and leukemias stain with a characteristic fluorescence intensity, it was possible to detect shifts in the proportions of $\kappa^+$ to $\lambda^+$ cells at discrete levels of fluorescence intensity even when total numbers of $\kappa^+$ and $\lambda^+$ cells were normal. The present report describes the detection of putative tumor cells in the peripheral blood of patients with malignant lymphoma even though the blood appears normal by classical cytologic criteria.

MATERIALS AND METHODS

Antisera

Rabbit sera specific for human $\kappa$ or $\lambda$ light chains (Behring Diagnostics, La Jolla, Calif.) were purified by affinity chromatography. Anti-gamma ($\gamma$) antiserum was prepared by immunization of rabbits with isolated $\gamma$ chains from pooled serum IgG. The antiserum contained anti-$\gamma$-chain antibodies, which were cross-reactive with mu ($\mu$) heavy chain, and the serum was subsequently absorbed with IgM, bound to Sepharose. F(ab')$_2$ fragments were prepared by digestion of IgG preparations with pepsin. Antibodies directed against $\kappa$ or $\lambda$ were tested for specificity by radioimmunoassay and by immunofluorescence using CLL cell samples bearing either Ig ($\kappa$) or Ig ($\lambda$). Of the 16 CLls stained with the purified antibodies, 12 stained exclusively with anti-$\kappa$ and 4 stained exclu-
sively with anti-λ. The reagents were titrated for staining of normal peripheral blood lymphocytes by indirect immunofluorescence using fluorescein isothiocyanate labeled goat anti-rabbit IgG (FI-GAR Ig, fluorescence/protein ~ 1.4). The IgG fraction of goat anti-rabbit Ig serum contained antibodies against γ, κ, and λ.14 FI-GAR Ig alone did not bind to viable human leukocytes, since human cells have a low avidity receptor for goat IgG. All reagents were used under saturating conditions.

**Immunofluorescence Staining**

Lymph node, bone marrow, and spleen samples were obtained during autopsy of trauma victims or biopsy for diagnostic studies. Viable lymphocytes from biopsies and whole blood were purified by centrifugation over 9% Ficoll (Sigma), 33% Hypaque (Winthrop).20 Cells were washed with phosphate-buffered saline, pH 7.2, resuspended in RPMI medium: 1640 (Grand Island Biologicals) containing 20% fetal calf serum (Grand Island Biologicals), and incubated for 16–20 hr at 37°C to remove cytophilic Ig.21 Subsequent staining and washing were performed at 4°C in a balanced salt solution containing 0.2% sodium azide. Aliquots of the cells were resuspended for 20 min at 5 x 10^7/ml in anti-κ (0.02 mg/ml), anti-λ (0.01 mg/ml), or no primary antibody (control). Cells were then washed and resuspended with FI-GAR Ig (0.1 mg/ml) for 10 min. After a final wash, cells were resuspended in Isoton (Coulter Electronics, Hialeah, Fla.) and examined on the Becton-Dickinson FACS III.

**Cytofluorimetry**

The fluoresceinated cells were exposed to laser light of 488 nm at an intensity of 300 mW. Living cells were distinguished from dead cells by near-forward angle light scatter. The fluorescence signal was determined with a photomultiplier (Type 9524, EMI Gencom, Inc., Plainview, N.Y.) potential of 500 V and the signal amplified so that distribution of fluorescence intensities of normal B cells stained with anti-κ or anti-λ were equivalent. Fluorescence and scatter signals were standardized daily using glutaraldehyde-fixed chicken erythrocytes. In order to minimize error when counting the small portion of the population with high intensity staining, 3 x 10^7 viable cells per sample were processed.

The percentage of positive cells was obtained by assessing the fluorescence of cells in the scatter channels characteristic of viable small lymphocytes and assigning as positive all cells to the right of the first inflection point on the descending fluorescence curve, as described previously.23

**Hematologic Methods**

T lymphocytes were enumerated by the rosette assay of Kaplan and Clark.24 For histopathologic studies, lymph node and spleen biopsies and bone marrow aspirates were processed in formalin and B-5 fixatives and stained with hemotoxylin, eosin, and periodic acid-Schiff reagent. Complete blood counts were performed on the Coulter Model S, and differential counts were visually determined.

**RESULTS**

**Patients**

The present study focused on patients with non-Hodgkin’s lymphoma of the small or lymphocytic type by the Rappaport classification25 who had no obvious leukemia or history of leukemia. This group included 12 patients with nodular, poorly differentiated lymphoma (PDL,N) 5 patients with diffuse, poorly differentiated lymphoma (PDL,D) 2 patients with diffuse, well differentiated lymphoma (WDL,D), and 1 patient with diffuse lymphoma of intermediate differentiation (IDL,D).26 The only tumors excluded from this group were T-cell neoplasms identified by the E-rosette technique.24 All tissue sections were reexamined without knowledge of their identity to confirm the diagnosis and classification of the tumor.

Salient clinical details are summarized in Table 1. Although some patients had a diagnosis of lymphoma prior to this study, in most cases, samples were analyzed at the time of the initial diagnosis. Past therapy in the former cases ranged from none to radiation and/or chemotherapy. However, only 3 patients were undergoing therapy at the time of our analyses. Seventeen of the 20 patients had stage III or stage IV disease27 at the time of study; since most patients have advanced disease at initial diagnosis, 28

**Table 1. Summary of Lymphoma Patients With no Obvious Leukemia**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Original Diagnosis*</th>
<th>Treatment†</th>
<th>Recent Treatment†</th>
</tr>
</thead>
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<tr>
<td>PDL,N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.5 yr SE, RT, CVP</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>11 yr RT</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>2 mo None</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>5 days VP</td>
<td>Regressing</td>
<td>VP</td>
</tr>
<tr>
<td>5</td>
<td>3.5 mo CVP</td>
<td>Stable</td>
<td>CVP</td>
</tr>
<tr>
<td>6</td>
<td>3 mo SE</td>
<td>Stable</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>3 mo RT</td>
<td>Stable</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>1 mo None</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>4 yr RT, CVP</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>5 mo CVP</td>
<td>Stable</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>1 mo None</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>None None</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>PDL,D</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2 wk None</td>
<td>Advancing</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>5 yr RT,CVP</td>
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<td>None</td>
</tr>
<tr>
<td>15</td>
<td>7 days None</td>
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<td>None</td>
</tr>
<tr>
<td>16</td>
<td>2 yr C,P</td>
<td>Advancing</td>
<td>P</td>
</tr>
<tr>
<td>17</td>
<td>3 days None</td>
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<td>None</td>
</tr>
<tr>
<td>WDL,D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>None None</td>
<td>Stable</td>
<td>None</td>
</tr>
<tr>
<td>19</td>
<td>4.5 yr RT</td>
<td>Stable</td>
<td>None</td>
</tr>
<tr>
<td>IDL,D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1 mo None</td>
<td>Stable</td>
<td>None</td>
</tr>
</tbody>
</table>

*All patients listed above had a diagnosis of malignant lymphocytic lymphoma based on pathologic examination of a lymph node, bone marrow, or spleen biopsy. Duration of disease indicates the period between the first diagnostic biopsy and analysis of peripheral blood in this study.

†Treatments are abbreviated as follows: C, cyclophosphamide; V, vincristine; P, prednisone; RT, radiotherapy; SE, surgical excision of involved tissues.

‡Recent treatments are those within 2 mo of the date of the peripheral blood analysis.
the distribution by stage seemed to be representative of the natural history of such tumors.

The complete blood counts for this group of 20 patients are recorded in Table 2. Two patients (nos. 1 and 5) had an elevated leukocyte count, but no patient had an absolute lymphocytic count over 4000/μl; patients with less than 4000 lymphocytes/μl are designated "nonleukemic" in accordance with the usual clinical criterion for leukemia. Patient 9 had lymphocytes with atypical morphology in his peripheral blood, but neither these cells nor the lymphocytes from any of the other patients in this group could be identified as neoplastic by morphological criteria. Four patients (nos. 3, 12, 15, and 16) had an elevation in the percentage of monocytes to ≥10% of total leukocytes; only patient 12 had an absolute monocytosis. Platelets were less than 100,000/μl in only one patient (no. 16). None of the patients had a monoclonal serum immunoglobulin detectable by serum protein electrophoresis.

Three groups of individuals provided both positive and negative controls for these studies. The positive controls consisted of 8 patients with both mass disease and past or present leukemia. In this group were five patients with WDL,D, one with PDL,N, and one with IDL,D. As negative controls, tissues from healthy donors and autopsy material from trauma victims were analyzed. The second type of negative control was provided by the study of patients with disorders other than B-cell tumors, including 40 patients with acute granulocytic leukemia, acute lymphoblastic leukemia, Hodgkin's disease, mycosis fungoides, carcinoma, myelofibrosis, autoimmune disease, angioimmunoblastic lymphadenopathy, and viral or bacterial infections.

**Analysis of T- and B-Cell Populations**

The viable cell population obtained after centrifugation on a Ficoll-Hypaque gradient contained T cells, B cells, and monocytes as assessed by microscopy and rosetting assays. A comparison of the lymph node cells from lymphoma patients and healthy individuals showed a decrease in the proportion of T cells in lymph nodes infiltrated with tumor (mean 27 ± 21 compared to 37 ± 23 for normal controls). Low to normal numbers of T cells were found in the peripheral blood of patients with lymphoma (50 ± 25 compared to 75 ± 10 for normal controls). The E-rosette data were used to ascertain that none of the 20 lymphoma patients had a T-cell lymphoma and that appropriate numbers of cells were stained with the anti-light-chain reagents (data not shown).

Approximately equal numbers of B cells stained with each of the anti-light-chain reagents in patients with disorders other than B-cell tumors and in normal controls (Fig. 1). The difference between the percentages of λ+ and μ+ cells in the entire population (ΔL) in these negative controls was 4% ± 3% for peripheral blood and 7% ± 6% for lymph nodes, spleens, and bone marrows. In these controls, no value of ΔL for blood cells exceeded 12% (mean ± 3 SD), and no value for other tissues exceeded 25% (mean ± 3 SD). In the group of patients with both lymphoma and leukemia, the ΔL was greater than these limits for 5 of 6 peripheral blood samples and 6 of 8 tissues infiltrated with tumor. For patients with lymphoma, but without leukemia, the ΔL exceeded these limits for 7 of 20 peripheral blood samples and 15 of 21 tumor tissues.

In addition to the Ig+ B cells and Ig− T cells, two other populations of cells were present in some samples to such an extent that the B-cell staining could not be evaluated by FACS analysis. The first of these populations, designated hereafter as "null cells," was identified as Ig− by FACS analysis and constituted a higher proportion of the total cells than could be accounted for by T lymphocytes as calculated from the E-rosette determination. In the controls, null cells predominated only in non-T, non-B-cell acute lymphoblastic leukemia and acute granulocytic leukemia. In nonleukemic patients with lymphoma, null cells were the...
DETECTION OF B CELL LYMPHOMA

A predominant population in two peripheral blood samples (nos. 8 and 18) and one bone marrow sample (no. 18). The second cell type stained weakly with anti-κ, anti-λ, and F(ab')2 anti-γ and is described in detail in the following section.

Effect of Monocytes on FACS Analysis

Most of the monocytes, which exhibited weak staining with both anti-light-chain reagents, were excluded from the FACS analysis on the basis of their distinct light scatter properties, as described in detail by Slease et al.3 Since the monocyte population was also stained using F(ab')2 anti-light-chain reagents and F(ab')2 anti-γ, the staining was due to cytophilic IgG from the donor's serum that remained on the monocytes even after overnight incubation. Described methods for removing cytophilic IgG, such as washing before centrifugation on Ficoll-Hypaque3 or brief exposure to acid3 were similarly unsuccessful.

The monocyte population identified by scatter and fluorescence was sorted and the identity confirmed by a cytologist, without knowledge of the source of the sample. This manipulation was not performed for each sample from the lymphoma patients, so that any unusual population of large lymphocytes with Fc receptors might also have been included in the “monocyte” category.

A slight overlap in the distributions of light scatter of monocytes and lymphocytes was often observed in the peripheral blood fractions. This overlap increased the total number of κ+ and λ+ cells but had no effect on the ΔL. Furthermore, the comparison between the numbers of κ+ and λ+ cells in the higher intensity channels is unaffected by minor contamination from monocytes, since all the cells staining with both anti-light-chain reagents have low intensity fluorescence and do not obscure more brightly stained B cells.

However, in some samples, Ig+ monocytes were the predominant population in all scatter channels, and the staining of B cells could not be analyzed. This was seen in the disease control group in samples from a patient with mycosis fungoides and from some patients with infections. In the group of lymphoma patients, monocytes predominated in one bone marrow (no. 20) and seven peripheral blood samples (nos. 7, 9, 12, 14, 15, 16, 20). In five of these seven peripheral blood samples, the ratio of lymphocytes to monocytes was less than 4 (Table 2). In the patients with high proportions of monocytes, F(ab')2 anti-γ stained the same number of cells as the anti-light-chain reagents,

Table 2. Analysis of Peripheral Blood of Lymphoma Patients

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Hematocrit</th>
<th>Platelets</th>
<th>White Blood Cell Count</th>
<th>Neutrophils*</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDL,N</td>
<td>1</td>
<td>Adequate</td>
<td>11,900</td>
<td>89</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>Adequate</td>
<td>10,100</td>
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<td>14</td>
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<tr>
<td>3</td>
<td>43</td>
<td>114,000</td>
<td>4,100</td>
<td>47</td>
<td>31</td>
<td>14</td>
<td>6</td>
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<td>4</td>
<td>40</td>
<td>290,000</td>
<td>7,400</td>
<td>96</td>
<td>4</td>
<td>0</td>
<td>0</td>
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<td>5</td>
<td>38</td>
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<td>15,300</td>
<td>72</td>
<td>23</td>
<td>5</td>
<td>0</td>
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</tr>
<tr>
<td>6</td>
<td>39</td>
<td>650,000</td>
<td>8,000</td>
<td>69</td>
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<td>7</td>
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<td>41</td>
<td>Adequate</td>
<td>7,000</td>
<td>76</td>
<td>17</td>
<td>4</td>
<td>0</td>
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<td>9,500</td>
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<td>41</td>
<td>230,000</td>
<td>6,200</td>
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<tr>
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<td>37</td>
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<td>72</td>
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<td>0</td>
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</tr>
<tr>
<td>PDL,D</td>
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<td>109,000</td>
<td>3,700</td>
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<td>32</td>
<td>2</td>
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<td>7,000</td>
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<td>17</td>
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<td>65</td>
<td>29</td>
<td>4</td>
<td>2</td>
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<tr>
<td>IDL,D</td>
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<td>9,100</td>
<td>52</td>
<td>44</td>
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ND, not determined.

*Neutrophils include both segmented and band forms.

†Adequate means platelets were not counted but judged as normal on the smear.
indicating that the polyclonal staining pattern was due to cytophilic serum IgG rather than binding of reagents (data not shown).

A variety of methods were used to remove monocytes from the peripheral blood cells, but none was entirely satisfactory. The use of two-color fluorescence to distinguish \( \kappa^+ \lambda^- \) monocytes from B cells by FACS analysis is currently under development.

**Determination of Clonal Excess**

In the peripheral blood samples from 13 of the 20 patients with lymphoma, nearly equal numbers of \( \kappa^+ \) and \( \lambda^+ \) cells were evident by FACS analysis. However, in some of these samples, lymphocytes bearing only the light-chain type of the tumor cells could be distinguished on dot-plot displays. We therefore devised a simple expression, clonal excess (CE), to distinguish between the numbers of \( \kappa^+ \) and \( \lambda^+ \) cells within discrete ranges of fluorescence intensity:

\[
\text{CE} = \frac{(\text{No. } \kappa^- \text{ cells}) - (\text{No. } \lambda^- \text{ cells})}{(\text{No. } \kappa^+ \text{ cells}) + (\text{No. } \lambda^+ \text{ cells})}
\]

The CE was calculated for every 10 channels of fluorescence intensity up to channel 200. The CE for channels 200–255 was calculated as a unit because the number of cells in these channels was low. Thus, a positive CE value reflects an excess of \( \kappa^+ \) or \( \lambda^- \) cells at any fluorescence intensity. By contrast, \( \Delta L \) expresses the difference between the total number of \( \kappa^+ \) and \( \lambda^- \) cells.

The CE profiles for five lymph node and peripheral blood samples from normal individuals are shown in Fig. 2. The CE values vary between —0.4 and 0.4 at all fluorescence intensities. By contrast, in tissues derived from lymphoma patients, the CE values could be calculated for 19 of 21 samples and were outside the normal range in all cases (Figs. 1 and 3). The 21 samples included 14 lymph nodes, 1 spleen, 2 mesenteric masses, 2 pleural fluids, and 2 bone marrows. In only 2 of these samples, both bone marrow aspirates, it was not possible to determine CE because of a predominant population of \( \kappa^+ \lambda^- \gamma^- \) or a low proportion of Ig cells. CE profiles for tumor cells from infiltrated tissues from five representative nonleukemic patients with \( \kappa^+ \) lymphomas are shown in Fig. 3. For all \( \kappa^+ \) lymphomas, the CE values are greater than 0.4 in fluorescence channels characteristic of the staining intensity of the tumor. For patients with \( \lambda^- \) tumors, the CE was less than —0.4 in the appropriate channels.

We analyzed peripheral blood mononuclear cells from all 20 nonleukemic patients with lymphoma. In all of the 11 samples for which the CE could be calculated, the CE was outside the normal range of the controls. Of these 11 patients, 3 (nos. 2, 3, and 11) had normal numbers of \( \kappa^+ \) and \( \lambda^- \) cells and a \( \Delta L \) less than 12% (Fig. 1). The CE profiles for peripheral blood mononuclear cells from 5 representative lymphoma patients without leukemia are shown in Fig. 3. In all patients with abnormal blood CE profiles, the predominant light-chain type on blood and tissue lymphocytes was the same. The CE could not be calculated for 7 blood samples due to monocyte interference and for 2
blood samples due to lack of Ig⁺ cells (presence of null cells).

The CE was plotted for all positive and negative control samples. In the group of lymphoma patients with leukemia, the shift in the CE plot was apparent in all lymph node, bone marrow, and peripheral blood samples (Fig. 1). The shift was prominent at high fluorescence intensities in tissues from patients with PDL,N and IDL,D. In no case did the CE profiles of blood or other tissues from patients with non-B-cell disorders resemble the profiles from patients with lymphoma.

Sensitivity of Clonal Excess Parameter

To determine the minimum number of tumor cells required to shift the CE profile out of the normal range, monoclonal lymphocytes from the blood of each of 3 patients with CLL or from an infiltrated node of a patient with PDL,D (2 separate experiments) was mixed with normal peripheral blood lymphocytes in varying proportions and stained with anti-light-chain reagents as described in Materials and Methods. Abnormal CE profiles were apparent in mixtures of cells containing as few as 1% CLL cells and 0.1% lymphoma cells (Fig. 4). The brightly staining lymphoma cells were more easily detected than the weakly fluorescent CLL cells because the number of normal cells in each group of ten channels decreases as the fluorescence intensity increases. We conclude from the above analysis that Ig⁺ tumor cells present at levels of 0.1%–1.0% in normal peripheral blood would be detectable by this method of measuring clonal excess and that detection is possible at any level of fluorescence intensity.

Donor Serum Does Not Induce a Clonal Excess in Normal Peripheral Blood

To eliminate the possibility that cytophilic immunoglobulin from the donor’s serum or a serum factor was
responsible for the deflection of the CE profiles of patients with lymphoma, normal peripheral blood lymphocytes were incubated for 1 hr at 37°C in undiluted serum from lymphoma patients whose blood showed CE. Cells were then incubated overnight at 37°C in RPMI-1640 containing 20% fetal calf serum, stained with anti-light-chain reagents and analyzed on the FACS. For these experiments, serum was selected from one patient (no. 17) with PDL,D whose tumor cells stained brightly with anti-κ and from another patient with WDL,D and CLL whose lymph node and peripheral blood cells stained weakly with anti-κ. In neither case did exposure to the patient’s serum cause a shift in the CE profile of the normal peripheral blood lymphocytes. These results indicate that neither cytophilic binding of monoclonal serum Ig nor other effects of serum factors are likely to cause the CE measured in patients with lymphoma.

Disappearance of Clonal Excess During Remission

To provide indirect evidence that the monoclonal cells detected in the peripheral blood are tumor cells and to determine their relationship to the activity of the disease, serial blood studies were performed on four patients, and the CE profiles were compared to changes in clinical status. These serial studies are summarized below.

Case 1. Patient 1 (Tables 1 and 2) developed a parotid mass 8 yr prior to study which, on biopsy, revealed “lymphosarcoma.” The mass and adjacent cervical lymph node were removed, and she received involved field radiotherapy. Two years prior to study, her enlarged spleen was removed. One year prior to study, she developed pleural effusions and was treated with 3 courses of cyclophosphamide, vincristine, and prednisone (CVP). Two months after treatment was discontinued, a lymph node was biopsied because of disease: histologic examination revealed PDL,N. Her leukocyte count at this time was elevated (Table 2) but her lymphocyte count was normal. FACS analysis of the lymph node and peripheral blood samples disclosed a CE of κ* cells (Fig. 3). Treatment with CVP was re instituted 2 wk after the biopsy and continued through 10 courses. All subsequent blood samples showed normal lymphocyte counts. Peripheral blood cells analyzed following six courses of CVP showed no CE. After the ninth course, a borderline CE was detected in her peripheral blood. Clinical examination revealed a small (0.5 cm) submental node that showed no change in the subsequent 6 mo. Peripheral blood analysis repeated 4 mo posttherapy showed no CE, but the levels of normal B cells were low and the proportions of T cells and monocytes were markedly increased in comparison to previous determinations. A chest roentgenogram revealed a right middle lobe pulmonary infiltrate, which on lung biopsy showed noncaseating granulomas. She was then placed on antituberculous chemotherapy and maintained a stable state for at least 4 mo thereafter.

Case 2. Patient 6 (Tables 1 and 2) was diagnosed as having a lymphoma coincident with the excision of a parotid node with a PDL,N lesion. Complete clinical evaluation revealed stage IA disease. The patient refused radiation and/or other postoperative therapy. A blood sample analyzed 6 wk after surgery showed a CE of κ* B cells. Four subsequent assays over the following year showed no CE. She has received no additional therapy, remains asymptomatic, and appears to be in complete remission.

Case 3. Patient 13 (Tables 1 and 2) presented with clinical stage III disease, which on biopsy was PDL,D. FACS analysis revealed a κ CE within the small cell population of his node and blood samples prior to therapy. After 5 mo of chemotherapy, he continued to show clinical evidence of lymphoma (splenomegaly), but his leukocyte count and differential remained normal. FACS analysis of a sample of peripheral blood drawn at this time indicated that the CE disappeared from the small cell population, but a κ* population appeared in the high scatter channels usually occupied by polyclonally staining monocytes, blast cells, or the large cells of histiocytic lymphoma. He developed progressive multifocal leukoencephalopathy and died 10 mo after diagnosis. Laparotomy 2 wk prior to death revealed PDL with slight nodularity in a mesenteric lymph node. Autopsy was not performed.

Case 4. Patient 17 (Tables 1 and 2) was found to have PDL,D stage IV. The disease presented in the supraclavicular and abdominal regions. FACS analysis of his peripheral blood mononuclear cells at the time of diagnosis showed a CE of κ* cells (Fig. 3). He was treated with CVP with a regression of mass disease. Recurrent tumor was evident 5 mo later, and by 7 mo postdiagnosis, he had a large epigastric mass, expanding left supraclavicular, and mediastinal adenopathy, and a large left pleural effusion. FACS analysis of the blood showed a CE of κ* cells that was larger than the excess detected 7 mo earlier. Analysis of pleural fluid also revealed a CE of κ* cells.

Thus, for all four patients, the CE appeared to increase or decrease during serial observations in parallel with mass disease. This correlation suggests that the monoclonal population in the peripheral blood may be a barometer of tumor activity in certain patients with lymphoma.

DISCUSSION

Malignant lymphoma of the lymphocytic (small cell) type is commonly disseminated (i.e., clinical stage III or IV) at the time of initial diagnosis. Nevertheless, a leukemic phase, if noted at all in these patients, is usually a late finding even when significant bone marrow involvement exists.

Complete clinical remissions have frequently been achieved in these patients by a variety of chemotherapy programs. In spite of this therapeutic success, the natural history of these patients is that of recurrence of disease and inevitable progression of disease to death. These results are in sharp contrast to those patients with malignant lymphoma of the histiocytic (large cell) type where complete clinical remissions with therapy are associated with a significant “cure” rate.

This dilemma of short-term therapeutic responsiveness but inevitable relapse in the lymphocytic lymphomas can be related to activity of disease not previously identified. The current study exploited the monoclonality of these tumors and the capacity of the FACS to quantify immunofluorescence to detect very small
numbers of tumor cells in the blood of lymphoma patients, even though the peripheral blood was judged normal with respect to number and morphology of lymphocytes. This evidence of a "subclinical" leukemia as a common accompaniment of such lesions has not heretofore been recognized. These findings suggest that classical staging procedures and criteria of remission fail to recognize disseminated disease that persists in spite of the disappearance of the mass disease.

Cytofluorimetry has been used by others to detect tumor cells that cannot be distinguished morphologically from normal cells. Melamed et al.,37 using acridine orange stain, monitored the disappearance of CLL cells during treatment. Ault38 also used the FACS to analyze peripheral blood cells from patients with non-Hodgkin's lymphoma stained with anti-light chain reagents, but he used a cumulative distribution technique to compare anti-\(\lambda\) and anti-\(\kappa\) staining. This technique detects a monoclonal population only if it comprises at least 10% of the peripheral blood lymphocytes. When his technique was applied to the data from our sensitivity analysis, no monoclonal populations were detected. Because of the cumulative nature of the mathematical analysis, Ault's technique is less sensitive for the detection of brightly staining monoclonal populations characteristic of patients with poorly differentiated lymphoma.9 In 22 of 25 nonleukemic patients with lymphoma, the percent of abnormal cells, as calculated by Ault, was within the range found for normal and disease controls.38 The greater sensitivity of the CE technique is based on the evaluation of relative numbers of \(\kappa^+\) and \(\lambda^+\) cells at discrete levels of fluorescence. As few as 0.1% lymphoma cells may be detected among normal blood lymphocytes using the CE analysis, and 11 of 20 peripheral blood samples from nonleukemic patients with lymphoma have values outside the range of normal and disease controls.

Marked deflection of the CE profile appears to reflect the presence of a monoclonal B-cell population for the following reasons. (1) The deflection is observed in tissues in which infiltration with lymphocytic lymphoma is apparent on pathologic examination but has been found in normal tissues. (2) No deflection is seen in the presence of non-B-cell tumors, even though there are residual B cells staining with the anti-light-chain reagents. (3) In tissue involved in an inflammatory reaction, the CE remains close to 0 even though the presence of monocytes obscures evaluation of many B cells and thereby makes the analysis less complete. (4) The deflection of the CE profile is not caused by cytophilic immunoglobulin, since none of the patients with lymphoma have paraproteinemia. (5) Exposure of normal lymphocytes to a patient's serum has no effect, suggesting that clonal excess is not induced by serum factors and providing further evidence that cytophilic immunoglobulin does not generate the observed CE.

We suggest that the monoclonal populations detected in the peripheral blood of patients with lymphoma are tumor cells for three reasons. First, the monoclonal population in the peripheral blood bears the same light-chain type as cells from the solid tumor. Second, in cases where sufficient cells were obtained to analyze heavy-chain classes (data not shown), discrete populations of cells bearing identical heavy-chain phenotypes (\(\mu, \mu\alpha, \text{or } \gamma\)) could be discerned in the same scatter channels for both the infiltrated node and peripheral blood. Third, in patients who were followed over a period of time, the CE disappeared after the onset of remission. Patients whose blood exhibited an abnormal CE profile continued to exhibit symptoms of lymphoma. Conclusive evidence that we are detecting tumor cells awaits further studies, such as heterotransplantation of the isolated blood cells into nude mice or assay of the ability of such cells to form colonies in semisolid media. Such tests are in progress.

Clonal excess was detected in 11 of 20 peripheral blood samples and 19 of 21 involved tissues from patients with clinically evident lymphocytic lymphoma. These figures probably underestimate the true frequency of CE positives because monocytes or other cells with nonspecifically bound Ig prevented an accurate assessment of CE in the nonpositive samples. Techniques under development that distinguish small monocytes from B cells may reveal a clonal excess in an even higher proportion of patients.

The finding of a clonal excess in blood samples was not confined to patients with longstanding, extranodal, progressive, or untreated disease. However, longitudinal studies in four patients suggested a correlation of clonal excess with tumor load and clinical status, i.e., CE positivity appeared to relate to progression of the disease. Additional serial studies of this sort, extended over the prolonged course of disease often seen in these patients, will be necessary to establish definitively the relationship between "clonal excess" in blood and disease activity.

The potential importance of our findings is fourfold. (1) Blood samples can be used to screen patients for B-cell lymphomas. (2) The presence and number of circulating tumor cells in malignant lymphocytic lymphomas may predict subsequent fluctuations in disease activity. (3) Minimal disease can be detected in patients already diagnosed and undergoing treat-
ment as a guide to when treatment should be intensified, stopped, or reinstituted. (4) The FACS permits separation and further analysis of circulating and in situ tumor cells; differences between these two populations may yield clues to the mechanisms underlying dissemination of the tumors.

REFERENCES

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Detection of tumor cells in the peripheral blood of nonleukemic patients with B-cell lymphoma: analysis of "clonal excess"

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